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Functional groups of sialic acids involved in binding to siglecs (sialoadhesins) deduced from interactions with synthetic analogues

Sørge KELM[1], Reinhard BROSSMER[2], Rainer ISECKE[2], Hans-Jürgen GROSS[3], Karen STRENGE[1] and Roland SCHAUER[1]

[1] Institute of Biochemistry, University of Kiel, Kiel, Germany
[2] Biochemistry Centre Heidelberg (BZH), University of Heidelberg, Heidelberg, Germany
[3] Institute of Clinical Chemistry, University of Ulm, Ulm, Germany

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The siglecs, formerly called sialoadhesins, are a family of I-type lectins binding to sialic acids on the cell surface. Five members of this family have been identified: sialoadhesin, myelin-associated glycoprotein (MAG), Schwann cell myelin protein (SMP), CD22 and CD33. We have investigated the relevance of substituents at position C-9 and in the *N*-acetyl group of *N*-acetylneuraminic acid, using a series of synthetic sialic-acid analogues either on resialylated human erythrocytes or as free α-glycosides in hapten inhibition. All five siglecs require the hydroxy group at C-9 for binding, suggesting hydrogen bonding of this substituent with the binding site. Remarkable differences were found among the proteins in their specificity for modifications of the *N*-acetyl group. Whereas sialoadhesin, MAG and SMP do not tolerate a hydroxy group as in *N*-glycolylneuraminic acid, they bind to halogenated acetyl residues. In the case of MAG, *N*-fluoroacetylneuraminic acid is bound about 17-fold better than *N*-acetylneuraminic acid. In contrast, human and murine CD22 both show good affinity for *N*-glycolylneuraminic acid, but only human CD22 bound the halogenated compounds. In conclusion, our data indicate that interactions of the hydroxy group at position 9 and the *N*-acyl substituent contribute significantly to the binding strength.

Keywords: Sialic acid; siglecs; structure-function relationship; sialoadhesin; myelin-associated glycoprotein.

The sialoadhesins [1–3] are a distinct group of I-type lectins [4] within the immunoglobulin superfamily (IgSF). Recently, a new name for this family has been proposed [5]; it accounts for their specificity for glycans carrying terminal sialic acids (Sia) and their structural properties as Ig-like proteins and lectins. Members of this family are sialoadhesin (Sn), siglec-1 [6, 7], expressed by tissue macrophages; CD22, siglec-2 [8–10], a B-cell-specific protein; CD33, siglec-3 [11], a marker for myeloid precursor cells; and myelin-associated glycoprotein (MAG), siglec-4a [12] and Schwann cell myelin protein (SMP), siglec-4b [13], both found in the myelin of peripheral and central nervous systems. Whereas CD22 strongly prefers glycans terminating in Siaα2→6Gal, the other members of the family bind to Siaα2→3-Gal-terminating structures.

Several biological functions for siglecs have been proposed. MAG seems to play a role in the organization of myelin, since mice develop an aberrant morphology of myelinated axons if the gene has been knocked out by homologous recombination [14–17]. In addition, MAG has been discussed as one of the components of myelin responsible for neurite-outgrowth inhibition [18–22]. The phenotype of CD22-deficient mice has provided good evidence that CD22 is involved in the regulation of B cell-dependent immune responses [23–26]. For Sn, a role in the regulation of interactions between tissue macrophages and myeloid cells has been assumed, since Sn binds preferentially to myeloid cells from all stages of maturation [27] and it is highly concentrated at the contact areas between macrophages and developing myeloid cells in the haematopoietic clusters of bone marrow [28]. At least Sn and MAG can function as cell-adhesion molecules in their native environment on macrophages or oligodendrocytes; this could not be demonstrated for CD22 on B-cells or CD33 on myeloid cells. In these cases, binding to glycoconjugates on the same cell (*cis* interactions) is likely to occur [29, 30].

Sia occur on cell surfaces at exposed positions, mostly as terminal components in different linkages to the glycans of glycoconjugates. A unique feature of Sia is their structural variability leading to more than 40 naturally occurring modifications. Therefore, Sia are thought to play important roles in cellular interactions [3, 31–35]. However, only a few proteins have been described, so far, that can be expected to function by binding to sialylated glycans *in vivo*. Probably, most prominent examples

Correspondence to Dr S. Kelm, Biochemisches Institut, University of Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany
Fax: +49 431880 2238.
E-mail: skelm@biochem.uni-kiel.de
URL: http://www.uni-kiel.de:8080/Biochemie/kelm/index.html
Correspondence to Prof. R. Brossmer, Biochemie-Zentrum, Universität Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany
Fax: +49 6221 545586.

Abbreviations. CHO, Chinese hamster ovary; Gc, glycolyl; IC_{50}, concentration required for 50% inhibition; IgSF, immunoglobulin superfamily; MAG, myelin-associated glycoprotein; Neu2en5Ac, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; NaCl/P/BSA, NaCl/P, (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.25% bovine serum albumin; rIP, relative inhibitory potency; Sia, sialic acid(s); SMP, Schwann-cell myelin protein; Sn, sialoadhesin; abbreviations used for synthetic Sia derivatives are compiled in Fig. 1.

Enzymes. Sialidase (EC 3.2.1.18); Galβ1→4GlcNAc α2→6-sialyltransferase (EC 2.4.99.1); Galβ1→3GalNAc α2→3-sialyltransferase (EC 2.4.99.4); Galβ1→3(4)GlcNAc α2→3-sialyltransferase (EC 2.4.99.6).

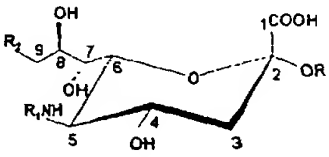
are the selectins, which mediate the rolling of leucocytes on endothelia [33, 36]. Sia-binding lectins from plants and animals, such as *Sambucus nigra* agglutinin, *Maackia amurensis* agglutinin and *Limax flavus* agglutinin, have been widely used as tools to detect sialylated glycans on cells or glycoconjugates.

Little is known about the molecular mechanisms relevant for the specific binding of sialylated glycans in comparison with other protein-carbohydrate interactions [37]. Probably, the best studied example is the haemagglutinin of influenza-A virus. In this case, the interactions between protein and carbohydrate have been studied intensively at the atomic level by crystallographic analysis of the three-dimensional structure of the protein complexed with the cognate ligand in combination with different binding assays [3, 38–41]. These experiments have demonstrated that especially the carboxyl group at C-1, the *N*-acetyl substituent at position 5 and the hydroxyl at position 8 contribute to the binding strength.

Less is known about the relevant interactions leading to the specific binding of Sia by other proteins [35]. With the exception of wheat-germ agglutinin, all Sia-binding proteins require the negatively charged carboxyl group for recognition. The necessity of an intact, unmodified extracyclic glycerol side chain (C-7 to C-9) varies from essential, as in the case of the siglecs [2] and the agglutinins of *Sambucus nigra* or *Limax flavus* [35], to dispensable, as for the agglutinins from *Maackia amurensis* [35], *Cepaea hortensis* [42], *Tachypleus tridentatus* [43] or the selectins [35]. The latter proteins even tolerate the replacement of Sia by other negatively charged residues, such as sulphate [44–46].

All members of the siglec family share similar structural features not commonly found in the IgSF [2, 47], i.e. an unusual V-set domain at the N-terminus and one (CD33) to sixteen (Sn) C-2-set domains, followed by the membrane anchor and the cytoplasmic C-terminus. Characteristic of all siglecs is an intrasheet disulphide between the B and F β-strands and a putative inter-domain disulphide bridge between the first two N-terminal domains. For Sn [48] and CD22 [49], the binding site has been mapped to the GFCC'-face of the V-set domain by site-directed mutagenesis. In the centre of this site is an arginine residue, which is found in all members of the siglec family. The high sequence similarities in this area support the idea that it represents the binding site also in MAG, SMP and CD33. In agreement with this, also for MAG, the corresponding Arg residue seems to be necessary for the binding of sialylated glycans [50].

It is important to note that all binding properties of the siglecs studied so far can be accounted for by binding to sialylated glycans, strongly suggesting that these Sia-dependent interactions are also important for their biological functions *in vivo*. Therefore, the molecular mechanisms responsible for the specific binding of Sia by these proteins are of major interest. The aim of this study was to investigate the contribution of structural features of Sia to the interaction with the siglecs. From previous experiments, it could be concluded that the substituents at positions 5 and 9 are critical for good binding by these proteins [49, 51–54]. Therefore, most chemical modifications of the 18 different Sia used were made in these positions. The results obtained provide evidence for the molecular mechanisms involved in Sia recognition and indicate possible strategies for the design of small-molecular-weight inhibitors, specific for each member of the siglec family.



	Aglycon R	Substituent R_1	Substituent R_9	Compound (abbreviation)
A	αC_2H_5	CH_3CO	OH	Neu5AcαBe (1)
	αCH_3	CH_3CO	OH	Neu5AcαMe (2)
	βCH_3	CH_3CO	OH	Neu5AcβMe (3)
B	-RBC	HCO	OH	Neu5Form (4)
	αCH_3	CH_3CH_2CO	OH	Neu5PropαMe (5)
	-RBC	OH-CH_2CO	OH	Neu5Gc (6)
	αCH_3	NH_2-CH_2CO	OH	Neu5NH$_2$AcαMe (7)
	αCH_3	FCH_2CO	OH	Neu5FAcαMe (8)
	αCH_3	$ClCH_2CO$	OH	Neu5ClAcαMe (9)
	-RBC	$BrCH_2CO$	OH	Neu5BrAc (10)
	αCH_3	CF_3CO	OH	Neu5F$_3$AcαMe (11)
	αCH_3	CH_3CS	OH	Neu5ThioAcαMe (12)
C	αCH_3	CH_3CO	H	9-deoxy-Neu5AcαMe (13)
	αCH_3	CH_3CO	Cl	9-Cl-Neu5AcαMe (14)
	αCH_3	CH_3CO	Br	9-Br-Neu5AcαMe (15)
	αCH_3	CH_3CO	I	9-I-Neu5AcαMe (16)
	αCH_3	CH_3CO	NH_2	9-NH$_2$-Neu5AcαMe (17)
	αCH_3	CH_3CO	SH	9-thio-Neu5AcαMe (18)

Fig. 1. Sialic acid analogues used in this study. All derivatives are based on neuraminic acid (Neu) shown at the top of the figure. Listed are the compounds with their abbreviations, following the suggestions by Reuter and Schauer [78], containing the aglycons R (A), the substituents R_1 at the C-5 amino group (B) and R_9 at C-9 (C). The modifications of interest are marked with bold letters. Compounds indicated by the aglycon-RBC have been tested with derivatized erythrocytes only.

EXPERIMENTAL PROCEDURES

Reagents. Sialidase from *Vibrio cholerae* was purchased from Behringwerke, carrier free $Na^{125}I$ from Amersham, affinity-purified anti-human IgG, Fc-specific antibodies from Biodesign. 2-Deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu2en5Ac) was from Boehringer Mannheim. For resialylation experiments, the following sialyltransferases were used to obtain cells containing exclusively Sia in the corresponding sialylated terminal oligosaccharide structures: Galβ1→4GlcNAc α2→6-sialyltransferase giving Siaα2→6Galβ1→4GlcNAc was purified from rat liver [55] and Galβ1→3GalNAc α2→3-sialyltransferase giving Siaα2→3Galβ1→3GalNAc structures was purified from porcine liver [56]. Recombinant Galβ1→3(4)GlcNAc α2→3-sialyltransferase [57] giving Siaα2→3Galβ1→3(4)GlcNAc was kindly donated by Dr J. C. Paulson at Cytel Inc., La Jolla, USA.

Fc-chimeras. Plasmids coding for the Fc-chimeras containing either the N-terminal one, three or four domains of Sn (Fc-Sn_{d1}, Fc-Sn_{d1-3} or Fc-Sn_{d1-4}, respectively), the N-terminal three or five domains of MAG (Fc-MAG_{d1-3} or Fc-MAG_{d1-5}) or the N-terminal three domains of murine CD22 (Fc-muCD22$_{d1-3}$) have been described [1, 58]. The plasmid coding for the Fc-chimera containing the N-terminal three domains of human CD22 [59] (Fc-huCD22$_{d1-3}$) was a kind gift from A. Varki, San Diego, USA. Fc-chimeras were produced by transient expression of the plasmids in COS cells, followed by purification from the tissue-culture supernatants by immunoaffinity chromatography on protein-A agarose [60]. Chinese hamster ovary (CHO) cells stably expressing MAG or SMP were kindly provided by Dr M. Filbin, New York, and Dr M. Tropak, Toronto, respectively.

Synthetic sialosides. In this study, 18 [redacted]des were used (Fig. 1). Neu5AcαBn (1) and Neu5AcαMe ([redacted]ere prepared by reaction of the 2-chloro derivative of peracetylated methyl *N*-acetylneuraminate with the respective alcohol employing molecular sieves [61]. Neu5AcβMe (3) was obtained as described previously [62]. The synthesis of Neu5PropαMe (5), Neu5thio-AcαMe (12) and 9-NH_2-Neu5AcαMe (17) has also been described [63]. Reaction of neuraminic acid methyl α-glycoside [63] with 4-nitrophenyl fluoroacetate, 4-nitrophenyl chloroacetate or ethyl trifluoroacetate afforded Neu5FAcαMe (8), Neu5-ClAcαMe (9) and Neu5F_3AcαMe (11), respectively. 9-Cl-Neu5-AcαMe (14), 9-Br-Neu5AcαMe (15) and 9-I-Neu5AcαMe (16) were synthesized by a Mitsunobu-type reaction, which will be reported in detail elsewhere and characterized by ^{1}H NMR spectroscopy and fast-atom bombardment MS. 9-Deoxy-Neu5AcαMe (13) was prepared by catalytic hydrogenation of 9-I-Neu5-AcαMe (16). 9-Thio-Neu5AcαMe (18) was obtained as described [64]. Preparative enzymatic syntheses of CMP-activated sialic-acid analogues were performed at pH 9.0, as outlined previously [61, 65]. Neu5BrAc (10) was activated at pH 7.5 to prevent hydrolysis of the Br atom. CMP-5-*N*-fluoresceinylneuraminic acid was prepared as described [66]. CMP-Neu5Gc was a kind gift from Dr L. Shaw, Kiel University, Germany.

Erythrocytes. Human erythrocytes were obtained from heparinized blood, washed, sialidase-treated and resialylated as described in previous studies [1, 51, 60]. Briefly, sialidase-treated erythrocytes were resialylated by incubation with one of the purified sialyltransferases in the presence of 750 µM CMP-sialosides for 3 h at 37°C. To determine the incorporation of sialic acids, remaining free acceptor sites were estimated by transfer of 5-*N*-fluoresceinylneuraminic acid with the corresponding sialyltransferases to residual acceptor sites on glutardialdehyde-fixed erythrocytes, and the fluorescent label was quantified by flow cytometry in a Coulter EPICS XL [51, 66, 67].

Binding assays. All assays were done in triplicate and were repeated at least three times. Purified Fc-chimeras were radioiodinated to specific activities of 20–80 Bq · pmol^{-1} and complexed with equimolar concentrations of anti-human IgG to achieve maximum binding to cells as described [1, 51, 60]. For hapten inhibition assays, the volume was scaled down to reduce the amount of inhibitor used. Complexed ^{125}I-labelled Fc-chimeras in NaCl/P_i (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.25% bovine serum albumin and 0.02% NaN_3 (NaCl/P_i/BSA) (10 µl containing about 10^3 Bq ^{125}I) were mixed with an equal volume of the inhibitor (threefold concentration, in NaCl/P_i, pH adjusted to 7.4 if necessary) and incubated for 1 h at 4°C before 10 µl human erythrocytes (0.25–0.5%) were added. After an overnight incubation at 4°C, unbound radioactivity was removed by five washes with 200 µl NaCl/P_i/BSA and the cell-bound radioactivity estimated in a gamma counter. As controls, the binding to sialidase-treated human erythrocytes and to untreated cells in the absence of inhibitor was also estimated. The inhibition was calculated by taking the values for binding in the absence of inhibitor as 0% inhibition and for sialidase-treated cells as 100% inhibition. The concentrations leading to 50% inhibition (IC_{50} values) were estimated by plotting the inhibition against the final inhibitor concentration. The precise IC_{50} values of Fc-chimeras binding were dependent on the amount and quality of erythrocytes or radiolabelled Fc-chimeras used in an assay. For a better comparison, the relative inhibitory potencies (rIPs) of tested Sia analogues were calculated from the IC_{50} of a compound relative to the IC_{50} for this reference substance. For compounds giving less than 50% inhibition at the highest concentration tested, rIPs are not available and can be considered as smaller than the value that would have resulted from 50% inhibition at this concentration.

Table 1. Resialylation of human [redacted]ocytes with synthetic Sia. Sialidase-treated human erythrocyte[redacted] resialylated using the corresponding sialyltransferases and synthetic CMP-Sia and yielding the indicated structures. The amount of Sia transferred expressed as a percentage of acceptor sites available on sialidase-treated cells was estimated as described under Experimental Procedures; n.d., not done.

Glycan structure	Sialic acid (Fig. 1)	Incorporation (% of acceptor sites)
Siaα2→6Galβ1→4GlcNAc	Neu5Ac	50
	9-deoxy-Neu5Ac	50
	Neu5Gc	60
	Neu5NH_2Ac	20
	Neu5Form	70
	Neu5BrAc	40
	Neu5ClAc	40
Siaα2→3Galβ1→3(4)GlcNAc	Neu5Ac	65
	9-deoxy-Neu5Ac	80
	Neu5Gc	45
	Neu5NH_2Ac	n.d.
	Neu5Form	60
	Neu5BrAc	85
	Neu5ClAc	85
Siaα2→3Galβ1→3GalNAc	Neu5Ac	80
	9-deoxy-Neu5Ac	90
	Neu5Gc	60
	Neu5NH_2Ac	55
	Neu5Form	85
	Neu5BrAc	85
	Neu5ClAc	90

These rIPs were reproducible, independent of the variable assay conditions, such as suboptimal complexing with anti-Fc antibodies.

RESULTS

Binding of siglecs to erythrocytes carrying different sialic-acid analogues. Fc-chimeras of siglecs bind to erythrocytes and other cells in a sialic acid-dependent manner, if complexed with the appropriate amounts of anti-Fc antibodies [1, 27, 60]. Sia on human erythrocytes were removed by sialidase treatment. Following this, naturally occurring or synthetic Sia were enzymatically incorporated as terminal components of defined glycans on the cells' surfaces. The amounts of Sia transferred by the individual sialyltransferases were estimated for each Sia analogue, as described under experimental procedures (Table 1), and the binding of Sn, MAG, murine and human CD22 as radioiodinated Fc-chimeras to these cells was estimated (Fig. 2). Independent of the type of Sia transferred, the linkage specificity observed for all four adhesins was in agreement with previous studies. Thus, Sn and MAG only bound to erythrocytes carrying α2→3-linked Sia, whereas CD22 bound only to cells with α2→6-linked Sia. It is important to note that high binding of MAG to Siaα2→3Galβ1→3(4)GlcNAc glycans could only be detected, if synthetic Sia analogues with halogenated *N*-acetyl groups, as in Neu5ClAc (9) or Neu5BrAc (10), had been transferred to the erythrocytes. This could be explained by a better binding of MAG to these Sia, as observed with monovalent Sia glycosides (see below). In addition, this demonstrates that MAG is able to bind Siaα2→3 linked to different underlying glycan structures.

None of the siglecs bound to erythrocytes carrying 9-deoxy-Neu5Ac (13) in any of the glycans resialylated (Fig. 2). This is

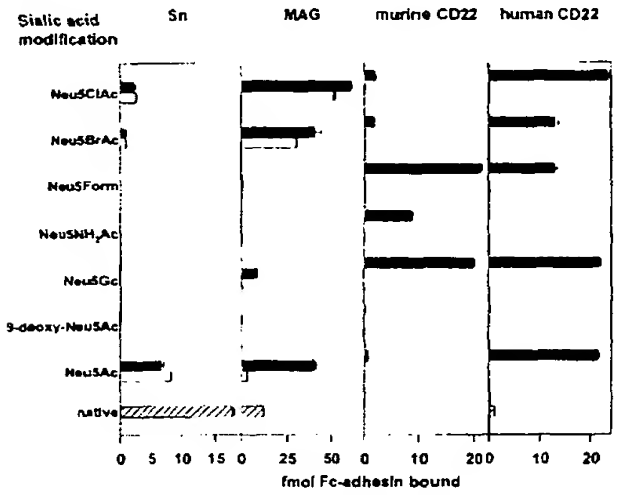


Fig. 2. Binding of radioiodinated Fc-chimeras to sialic acid analogues on erythrocytes. Human erythrocytes were derivatized to contain the Sia analogues indicated in one of the following linkages: Siaα2→6-Galβ1→4GlcNAc (black bars), Siaα2→3Galβ1→3(4)GlcNAc (white bars) or Siaα2→3Galβ1→3GalNAc (grey bars). Native erythrocytes (hatched bars) contain all three types of glycans. Binding to the cells was estimated using antibody-complexed Fc-chimeras (100 fmol in a total volume of 50 µl) of $Sn_{\alpha 1-3}$, $MAG_{\alpha 1-3}$, murine $CD22_{\beta 1-3}$ or human $CD22_{\alpha 1-3}$ in the fluid-phase assay described under Experimental Procedures. Note the different scale used for MAG binding to accommodate the high binding of this protein to Neu5ClAc and Neu5BrAc.

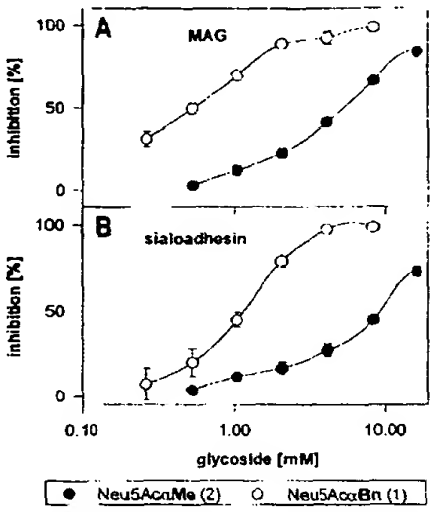


Fig. 3. Hapten inhibition with sialosides containing different aglycons. Native human erythrocytes were incubated with antibody-complexed Fc-$MAG_{\alpha 1-3}$ (A) or Fc-$Sn_{\alpha 1-3}$ (B) in the presence of the indicated glycosides at different concentrations. Binding and inhibition were determined as described for the hapten inhibition assay under Experimental Procedures.

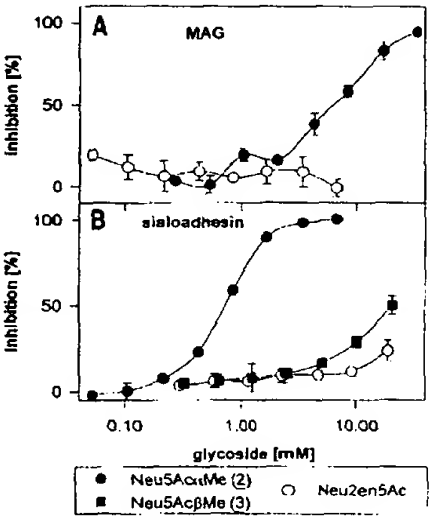


Fig. 4. The influence of the ring conformation and different anomeric configurations. Native human erythrocytes were incubated with antibody-complexed Fc-$MAG_{\alpha 1}$ (A) or Fc-$Sn_{\alpha 1-3}$ (B) in the presence of the indicated Sia derivatives at different concentrations. Binding and inhibition were determined as described for the hapten inhibition assay under Experimental Procedures. Compound (3) was not tested with MAG.

good evidence that the hydroxy group at this position is contributing significantly to the binding energy. Whereas Sn, CD22 and MAG had the same requirement of the hydroxyl at C-9, the interaction with Sia modified at the *N*-acetyl group varied significantly. Sn, MAG and human CD22 bound well to cells carrying Neu5Ac (2), whereas the murine homologue did not show significant binding to these cells. In contrast, only murine and human CD22 bound to Neu5Gc (6). Replacing the acetyl group with the shorter formyl residue (4) abolished binding of Sn and MAG, whereas both human and murine CD22 were able to bind cells carrying this Sia. Only murine CD22 showed significant binding to the aminoacetyl-derivative (7), although it was lower than to Neu5Gc (6).

The most dramatic differences in binding strength were found for the analogues with halogenated *N*-acetyl groups (Fig. 2). In particular, MAG bound best to erythrocytes carrying these analogues, leading to the binding of glycans with the structure Siaα2→3Galβ1→3(4)GlcNAc as well as Siaα2→3Galβ1→3GalNAc. The amount of MAG bound to cells containing the former glycan with Neu5ClAc was 17-fold higher than to those with Neu5Ac, whereas for Neu5BrAc the increase was 10-fold. In contrast, a lower, but still detectable binding of Sn and murine CD22 occurred to cells carrying Neu5ClAc or Neu5BrAc on suitable glycans, whereas the affinity of human CD22 for Sia does not seem to be affected by the introduction of the halogen atoms.

In order to compare the binding specificity of the closely related proteins MAG and SMP, qualitative cell-binding assays were also performed with CHO cells stably expressing these proteins. Since these cells produce high levels of α2→3-linked Sia, binding of erythrocytes could only be detected after sialidase treatment of the CHO cells to unmask the binding sites of the adhesion molecule [2, 53], as first described for CD22 [29, 68] and CD33 [30]. In all cases, SMP showed a specificity indistinguishable from that of MAG, reflecting the specificity found with the fluid-phase assay described above, i.e. CHO cell lines expressing either SMP or MAG bound erythrocytes carrying Neu5Ac, only if it was α2→3 linked to Galβ1→3GalNAc, whereas Neu5ClAc or Neu5BrAc were also bound if α2→3-linked to Galβ1→3(4)GlcNAc. Furthermore, erythrocytes containing Neu5ClAc or Neu5BrAc were also bound by CHO cells without prior sialidase treatment (data not shown). Taken to-

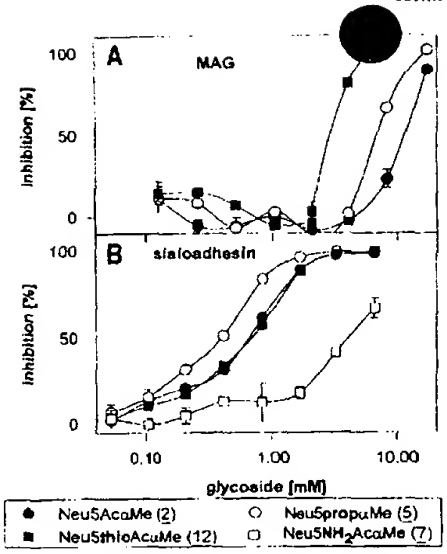


Fig. 5. The influence of modifications of the *N*-acyl group of sialic acid. Native human erythrocytes were incubated with antibody-complexed Fc-MAG_{d1} (A) or Fc-Sn_{d1-3} (B) in the presence of the indicated Sia derivatives at different concentrations. Binding and inhibition were determined as described for the hapten inhibition assay under Experimental Procedures.

gether, these results demonstrate that both MAG and SMP have a higher affinity for Sia with halogenated *N*-acetyl groups.

Hapten inhibition assay. Simple Sia glycosides inhibited the binding of antibody-complexed Fc-chimeras to human erythrocytes. None of the inhibitors used caused a lysis of erythrocytes. This inhibition was concentration dependent (Fig. 3). For MAG and Sn, Neu5AcαBn (1) was a more potent inhibitor than Neu5AcαMe (2), since (2) has an eight- to tenfold higher IC_{50} value. This finding suggests additional hydrophobic and/or steric interactions of the benzyl residue with the protein. In order to prevent such effects and to facilitate the comparison of different Sia analogues, in all further assays (2) was used as reference compound. The IC_{50} values obtained varied between different assays, since they were dependent on conditions such as the erythrocyte concentration. However, if the IC_{50} values of inhibitors were compared with those of the reference compound (2) tested in parallel in each assay, consistent rIP values were obtained, which also allowed a reliable comparison among different assays. Comparable rIP values were obtained for Fc-chimeras containing different numbers of N-terminal domains, e.g. Fc-MAG_{d1-3} versus Fc-MAG_{d1-5} or Fc-Sn_{d1} versus Fc-Sn_{d1-3}.

The main structural difference between the anomeric conformations of Sia is the position of the carboxyl group, which is axial in the α-anomer, the natural conformation in sialylated glycans. On the other hand, the strong sialidase inhibitor Neu2en5Ac has an altered ring conformation. We compared the inhibitory potency of Neu5AcαMe (2) with Neu5AcβMe (3) and Neu2en5Ac (Fig. 4). As shown for Sn, good inhibition of binding was only obtained with (2), whereas more than 20-fold higher concentrations of (3) were required for 50% inhibition. Furthermore, Neu2enAc was not inhibitory as shown for Sn and MAG. This demonstrates the importance of an axial position of the carboxyl group and the correct ring conformation for the binding of Sia by both proteins.

Modifications of the substituent at C-5. The binding assays with derivatized erythrocytes have demonstrated the importance

Table 2. Relative inhibitory potency of sialosides. The rIPs of each sialoside was calculated by dividing the IC_{50} of the reference compound Neu5AcαMe by the IC_{50} of the compound of interest. This results in rIPs above 1.0 for Sia derivatives binding better than Neu5AcαMe and rIPs lower than 1 for structures binding less than Neu5AcαMe. n.a., not applicable, less than 50% inhibition at the highest concentration tested (20 mM); n.d., not done.

Compound (Fig. 1)	rIP for Sn	rIP for MAG
Neu5AcαBn (1)	8.10	9.80
Neu5AcαMe (2)	1.00	1.00
Neu5AcβMe (3)	n.a.	n.d.
Neu5propαMe (5)	1.56	1.56
$Neu5NH_2Ac\ \alpha Me$ (7)	0.14	n.a.
Neu5FAcαMe (8)	1.67	16.94
Neu5ClAcαMe (9)	0.78	7.00
$Neu5F_3Ac\alpha Me$ (11)	1.40	4.04
Neu5thioAcαMe (12)	0.87	3.85
9-deoxy-Neu5Ac (13)	0.03	n.a.
9-Cl-Neu5Ac (14)	0.04	n.a.
9-Br-Neu5Ac (15)	n.a.	n.a.
9-I-Neu5Ac (16)	0.04	n.a.
$9\text{-}NH_2\text{-}Neu5Ac$ (17)	1.56	2.98
9-thio-Neu5Ac (18)	0.16	n.a.

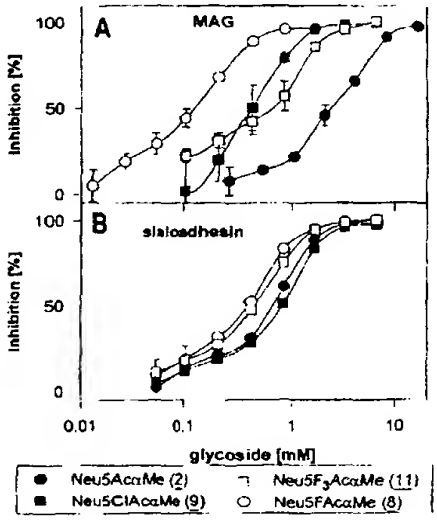


Fig. 6. The influence of halogen substitution of the *N*-acetyl group of sialic acid. Native human erythrocytes were incubated with antibody-complexed Fc-MAG_{d1} (A) or Fc-Sn_{d1-3} (B) in the presence of the indicated Sia derivatives at different concentrations. Binding and inhibition were determined as described for the hapten inhibition assay under Experimental Procedures.

of the *N*-acyl residue of Sia for recognition (Fig. 2). Therefore, we compared the inhibitory potencies of αMe-sialosides with modifications at this position (Fig. 1B) using Neu5AcαMe (2) as a reference compound. In one series, the acetyl residue was replaced with the propionyl (5), an aminoacetyl (7) or a thioacetyl (12) group (Fig. 5, Table 2). Whereas (5) was a slightly better inhibitor than (2) for Sn and MAG, the effect of the other modifications revealed differences between the two adhesins. The most significant enhancement was found with (12), which was a more potent inhibitor for MAG, but not for Sn. The introduction of an amino function, as in (7), prevented binding of Sn

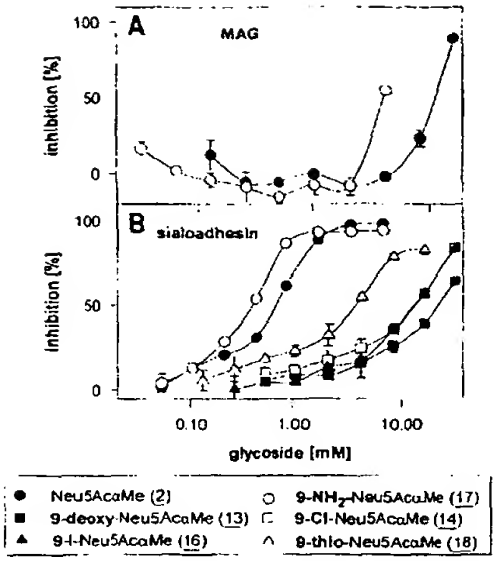


Fig. 7. The influence of substituents at C-9 of sialic acid. Native human erythrocytes were incubated with antibody-complexed Fc-MAG_{d1} (A) or Fc-Sn_{d1-5} (B) in the presence of the indicated Sia derivatives at different concentrations. Binding and inhibition were determined as described for the hapten inhibition assay under Experimental Procedures.

and MAG to derivatized erythrocytes (Fig. 2). As methyl-α-sialoside, this compound could inhibit Sn binding to erythrocytes, but required more than tenfold higher concentrations than (2) for equal inhibition. For MAG, no inhibition by this compound could be detected at concentrations up to 10 mM.

Based on the observation that halogenated acetyl residues strongly enhance the affinity of MAG and SMP for sialylated glycans on erythrocytes, compounds containing halogen atoms were compared with (2) (Fig. 6). For Sn, Neu5ClAcαMe (9) was as effective as the reference compound (2), and the inhibitory potencies of the fluor-containing compounds Neu5FAcαMe (8) and $Neu5F_3AcαMe$ (11) were only slightly higher. In contrast, the introduction of one chlor (9) or three fluor atoms (11) enhanced the affinity for MAG seven- or fourfold, respectively. By far the most potent inhibitor for MAG was Neu5FAcαMe (8) with a rIP of 17. These results strongly suggest that the electronegative halogens mediate an additional interaction especially with the binding site of MAG, leading to the higher affinities observed.

Modifications at C-9. Also for the hydroxy group at C-9 of Sia, interactions with the binding site of siglecs are very likely to contribute to the binding strength, as demonstrated by the experiments with derivatized erythrocytes (Fig. 2). Therefore, this function has also been replaced by several other substituents (Fig. 1). Hapten-inhibition experiments with these compounds demonstrated that the interaction of the hydroxyl function at C-9 with the binding sites of Sn and MAG could also be mediated by an amino function, since the corresponding compound 9-amino-Neu5AcαMe (17) was an even more potent inhibitor than the reference sialoside Neu5AcαMe (2) (Fig. 7). In contrast, the introduction of a thiol group, as in 9-thio-Neu5AcαMe (18), reduced the rIP for Sn more than fivefold. An even weaker binding was obtained for the halogenated compounds (14 and 16) or the 9-deoxy-analogue (13), for which the rIPs were more than 20-fold lower. No inhibition of Sn was obtained with 9-Br-Neu5AcαMe (15) at concentrations up to 20 mM. MAG was also not inhibited by the halogenated or deoxygenated derivatives at concentrations up to 20 mM.

DISCUSSION

The experiments with derivatized erythrocytes described here lead to the conclusion that the hydroxy group at C-9 and the *N*-acetyl residue at C-5 are essential structural features of Sia, recognized by members of the siglec family (Fig. 8). Erythrocytes resialylated with Sia in specific linkages have been powerful tools in previous studies of the specificities of the siglecs [1, 6, 30, 51, 58]. However, there are several limitations to this approach. For example, it is difficult to obtain good relative affinities for cell-surface sialosides. Therefore, in addition, a hap-

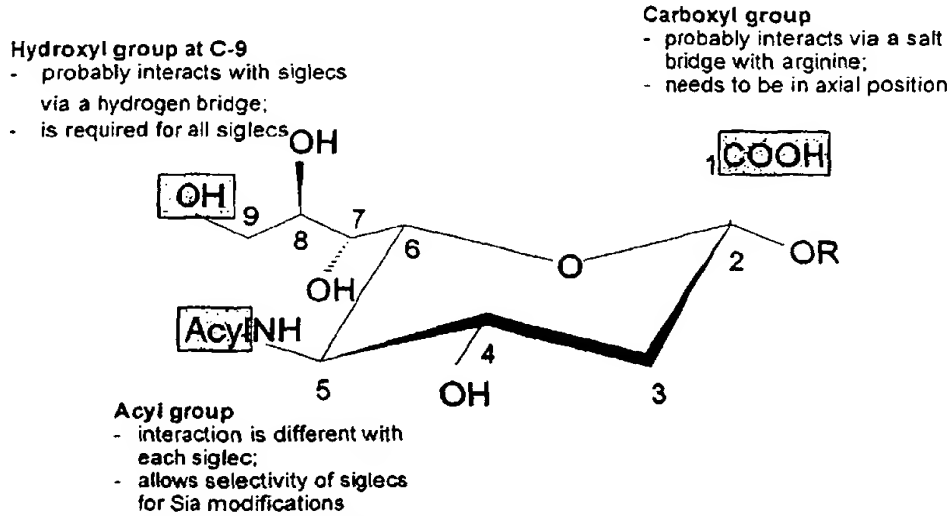


Fig. 8. Functional groups of sialic acid interacting with siglecs. The functional groups of Sia important for the binding by siglecs identified in this study are indicated by grey boxes.

ten inhibition assay for siglecs was developed, applying human erythrocytes as target cells. The rIPs calculated from inhibition curves (Table 2) allowed a quantitative comparison, from which conclusions can be drawn concerning the importance of several structural features of Sia for recognition by the siglecs. A similar approach has been useful in defining the relevance of interactions predicted to occur between the influenza virus haemagglutinin and Sia [38]. Since murine CD22 binds only weakly to human erythrocytes, only Sn and MAG were compared in the inhibition experiments.

The position of the carboxyl group is essential for the binding of Sia, suggesting possible interactions with the protein. By site-directed mutagenesis, the putative binding site on Sn [48] and CD22 [49] has been located on the *GFCC' β*-sheet of domain 1. In the centre of this region is an arginine residue. It is tempting to speculate that the arginine, which has been shown to be essential for Sia binding by Sn [48], CD22 [49] and MAG [50], is interacting with the carboxyl group of Sia. Also, for other Sia-binding proteins, such as various adhesins from bacteria [69] or the selectins [70], the basic amino acids lysine and arginine have been suggested to be involved in Sia binding. In the case of the influenza-A virus haemagglutinin, the requirement for an axial position of the carboxyl group has been demonstrated [38]. In this case, an interaction with an amide of the protein backbone and the hydroxy group of Ser_{136} has been proposed.

Although previous work [3, 51–54] pointed at a critical role of the glycerol side chain of Sia for recognition by the siglecs, it remained unclear whether single hydroxy groups were involved in the interactions of Sia with the binding site. The data presented here demonstrate the requirement of the hydroxy group at C-9 for binding, since 9-deoxy-Neu5Ac or the 9-halogenated analogues of Neu5Ac were not bound by any of the siglecs tested. This suggests an important contribution of this hydroxy group, such as a hydrogen bond with one or more amino acids in the binding pocket. Because an amino function at this position can supply the same interaction, at least in the case of Sn and MAG, it is likely that the hydroxyl at C-9 functions as a hydrogen donor, whereas electronegative halogen atoms cannot replace the hydroxy group. It is important to note that, compared with an amino group, a thiol at C-9 of Sia (18) interacts much more weakly, although a SH function can form hydrogen bonds both as donor or as acceptor for hydrogen. An explanation for this apparent discrepancy may be steric constraints by the large sulphur atom. Also the hydroxy group at C-8 plays a crucial role in the binding strength, whereas the hydroxyl at C-7 does not seem to be required (Strenge, K., Schauer, R., Bovin, N., Hasegawa, A., Ishida, H., Kiso, M. and Kelm, S., unpublished observations). These conclusions regarding the roles of hydroxyl groups in the glycerol side chain are in excellent agreement with the structure of Sn complexed with α2→3-sialyllactose, as determined by X-ray crystallography [71].

The role of the glycerol side chain for the recognition of Sia by some other Sia-binding proteins has been studied, mainly by mild periodate oxidation. For example, the selectins tolerate this modification of Sia without loss of binding [72–74]. In fact, the selectins apparently do not recognize Sia specifically, since it can be replaced by other negatively charges residues, such as sulphate [45, 72, 75]. Also, for the haemagglutinin of influenza-A virus, it has been shown that 9-*O*-acetylation of Sia prevents its binding [76]. However, this seems to be due to sterical hindrance, since the hydroxy group at C-9 is not necessary [38].

Acetyl or glycolyl residues are naturally occurring substituents of the amino function at C-5 in Neu5Ac and Neu5Gc, respectively. The observation that murine CD22 and Sn have opposite specificities for these Sia [51] was the first evidence that this region of Sia can modulate selective binding of cells. This has been confirmed and extended in this study, showing that each siglec has a distinct specificity profile for Sia with modifications of this substituent.

The most striking result obtained was the strongly enhanced binding of MAG to Sia containing halogenated acetyl residues. In hapten inhibition assays, Neu5FAc was the Sia with the highest rIP for MAG (Table 2). MAG even bound to Siaα2→3-Galβ1→3(4)GlcNAc glycans on resialylated erythrocytes, if the Sia incorporated was Neu5ClAc and Neu5BrAc, whereas it did not bind to this glycan if the Sia was Neu5Ac [1]. This is due to a stronger affinity of these Sia for MAG and SMP and not a general increase in stickiness, since such an enhanced binding was not obtained with the other siglecs. However, this higher affinity cannot overcome the specificity for α2→3-linked Sia, since Siaα2→6Galβ1→4GlcNAc glycans containing Neu5ClAc or Neu5BrAc could not mediate adhesion of erythrocytes to MAG or SMP. In addition, these data suggest that MAG and SMP also recognize the structure Siaα2→3Galβ1→3(4)GlcNAc.

Sn and MAG have in common a preference for Neu5Ac rather than Neu5Gc or $Neu5NH_2Ac$ and do not bind to Neu5-Form. In the case of $Neu5NH_2Ac$, the low affinity found for all proteins could be due to the formation of an inner salt, leading to a decrease of the negative charge of the molecule.

It seems reasonable to assume that in the binding sites of both proteins, amino acids interact specifically with the methyl group of the acetyl residue. In fact, in the structure of Sn crystals complexed with α2→3-sialyllactose, this methyl group is located over the aromatic ring of a tryptophan residue [71]. This is similar to the influenza-A haemagglutinin, where this methyl group has also been located over a tryptophan residue [41]. The enhanced affinity of Neu5FAc, Neu5ClAc and Neu5BrAc for MAG could be explained by strengthening such an interaction or by an additional contact with the protein mediated by the halogen atoms. It is important to note that this enhancement correlates with the electronegativity of the halogen. In particular, an effect on the amide structure of the acetyl residue has to be taken into account, resulting in a weaker hydrogen bond acceptor quality of the carbonyl oxygen and a significantly stronger hydrogen bond involving the amino group. In this context, it is interesting that a reduction of the electron density by replacing the carbonyl oxygen with a sulphur atom also caused an increased affinity for MAG. It has been shown previously that this chemical modification strongly enhanced the suppression of influenza-A virus infection by a *N*-thioacetylneuraminic acid acrylamide copolymer [77]. In this case, the effect has been explained in a similar way. In contrast to MAG, for Sn, no stronger binding of any of these Sia was found, suggesting that these effects are not relevant for the interaction with Sn.

Different effects of halogen atoms in the *N*-acyl residue were also detected. Murine CD22 seems to require a functional group that can be involved in hydrogen bonding, since $Neu5NH_2Ac$ could, at least in part, replace Neu5Gc, whereas it did not bind to Sia containing any of the halogenated acetyl groups. In contrast, the capability of the human CD22 to bind Neu5Ac correlated with the tolerance of halogenated *N*-acetyl groups, reflecting more of the situations found for MAG and Sn. During evolution, human CD22 had to develop an affinity for Neu5Ac, since, in contrast to most animals, humans do not express Neu5Gc [32]. For this, the requirement of interactions mediated by the hydroxy group may have been replaced by another contact, possibly with the methyl group similar to Sn and MAG. It is important to point out that murine CD22 also binds to erythrocytes containing Neu5Acα2→6Gal, if less-stringent assays are used [1, 51].

In conclusion, the data presented here demonstrate the specific recognition of Sia by members of the siglec family. Furthermore, Sia analogues, such as Neu5FAc, will be the basis for the development of analogues tailored for high affinities towards one member (MAG) of the siglec family. The information presented here, together with the three-dimensional structure of Sn complexed with 2→3-sialyllactose now available, will be helpful in defining the contributions of protein-carbohydrate contacts and in the development of models for the other members of the family.

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